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PATENT
Docket No.: **19603/468 (CRF D-1595C)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Barany et al.	Examiner: J. Ricigliano
Serial No.	:	08/794,851	Art Unit: 1627
Filed	:	February 4, 1997	
For	:	DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING THE LIGASE DETECTION REACTION WITH ADDRESSABLE ARRAYS	

DECLARATION OF FRANCIS BARANY UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, FRANCIS BARANY, pursuant to 37 C.F.R. § 1.132, declare:

1. I received a B.A. in Chemistry in 1976 from the University of Illinois at Chicago Circle and a Ph.D. in Microbiology from The Rockefeller University in 1981.

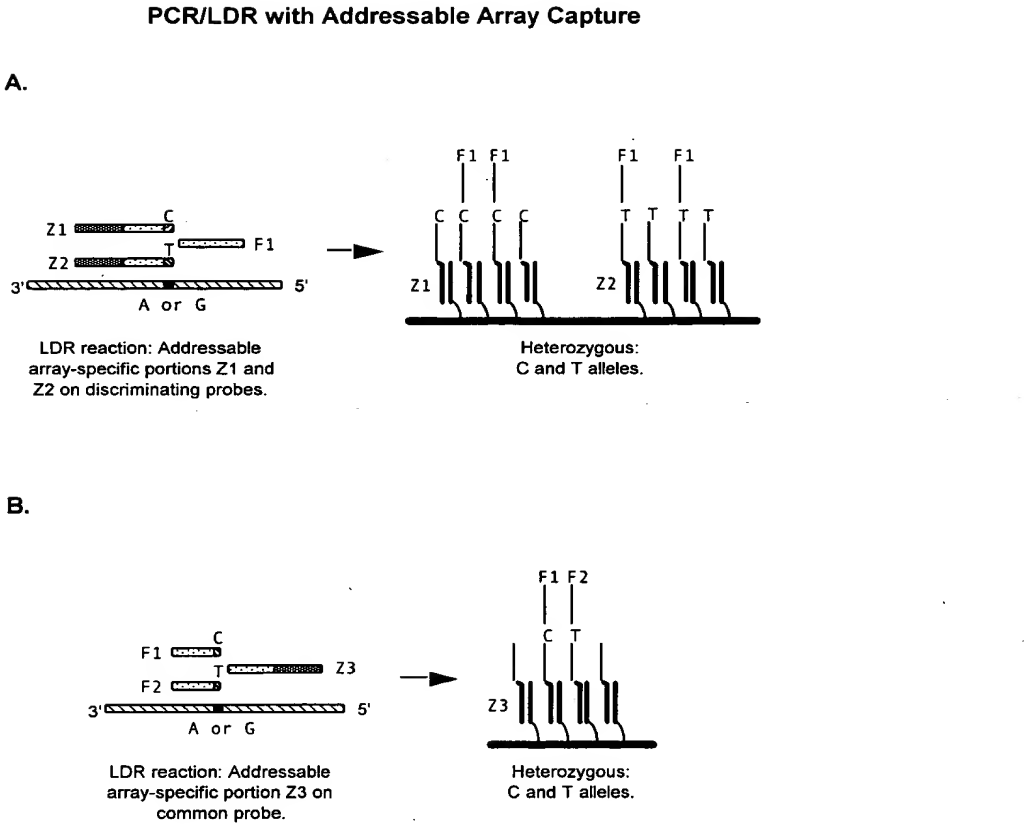
2. I am a Professor of Microbiology at the Joan and Sanford I. Weill Medical College of Cornell University, New York, New York.

3. I am an inventor of the above-identified patent application.

Ligase Detection Reaction/Universal Array Capture

4. The present application relates to a procedure of identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences by using the combination of ligase detection reaction ("LDR") and array capture on a solid support. An example of this procedure is depicted in Figure 1.

Figure 1



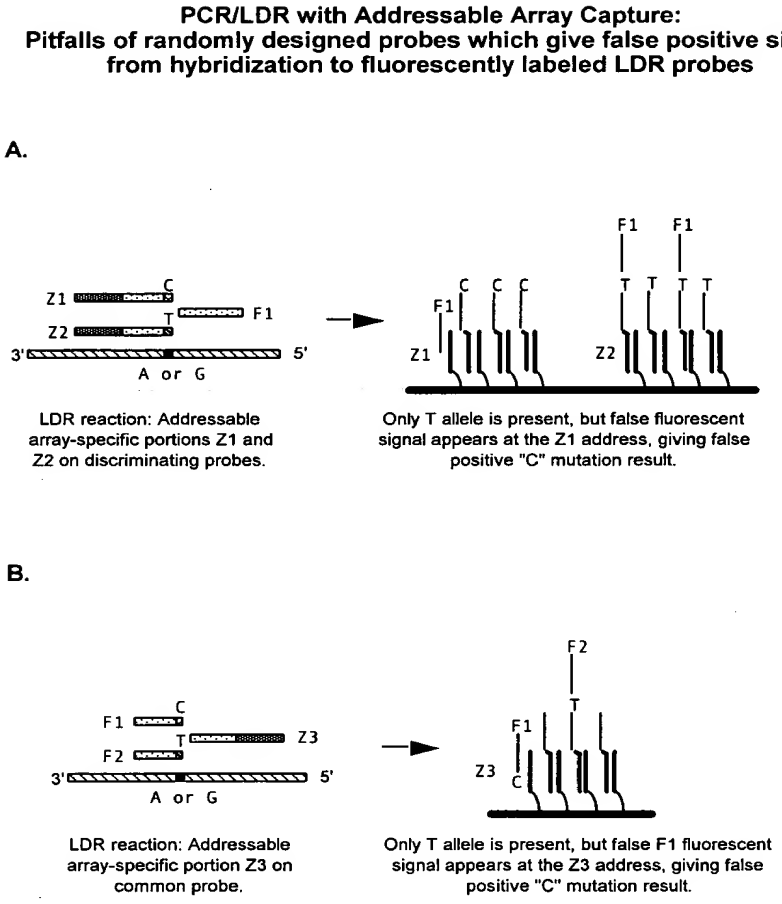
In Figure 1A, the two allele discriminating LDR probes "C" and "T" contain addressable array specific portions Z1 and Z2, respectively. The common probe contains fluorescent group F1. In the presence of target DNA and DNA ligase, only adjacent probes with perfect complementary at the junction can ligate, and the resultant product is captured on a universal addressable array. The presence of fluorescent signal F1 at address Z1 indicates presence of the C allele in the target nucleic acid, while the presence of fluorescent signal F1 at address Z2 indicates presence of the T allele in the target nucleic acid. In Figure 1B, the two allele discriminating LDR probes "C" and "T" contain fluorescent groups F1 and F2, respectively. The common probe contains addressable array specific portion Z3. As above, in the presence of target DNA and DNA ligase, only adjacent probes with perfect complementary at the junction can ligate, and the resultant product is captured on an addressable array. The presence of fluorescent signal F1 at address Z3 indicates presence of the C allele in the target

nucleic acid, while presence of fluorescent signal F2 at address Z3 indicates presence of the T allele in the target nucleic acid. Preferably, LDR is preceded by a polymerase chain reaction ("PCR") procedure.

5. The process of the present invention allows for the capture oligonucleotide to be placed on either side. Thus, one can use a unique address for each mutation, capturing the fluorescent signal for every mutation, even when present in an excess of wild-type DNA. This principle was demonstrated in working with the K-ras gene, where mutations were detected in the presence of a 200-fold excess of wild-type DNA (Gerry, N., Witowski, N., Day, J., Hammer, R., Barany, G. and Barany, F., "Universal DNA Array With Polymerase Chain Reaction/Ligase Detection Reaction (PCR/LDR) for Multiplex Detection of Low Abundance Mutations," J. Mol. Biol. 292:251-262 (1999)).

6. The capture oligonucleotide probe needs to be carefully designed for capture of an LDR ligation product to avoid false positive or false negative signals which would result from the random design used with the primer extension technique described below. A random design may be used on the primer extension approach, because the fluorescent label is on the dideoxynucleotide and, therefore, would not hybridize to any address on the array unless incorporated onto a (presumably correctly hybridized) probe. In contrast, with LDR, the fluorescent label(s) is (are) either on the discriminating probes or on the common probe. Incorrect hybridization of one of these to an address on the array would give a false positive signal, with potentially disastrous consequences. This is illustrated in Figure 2.

Figure 2

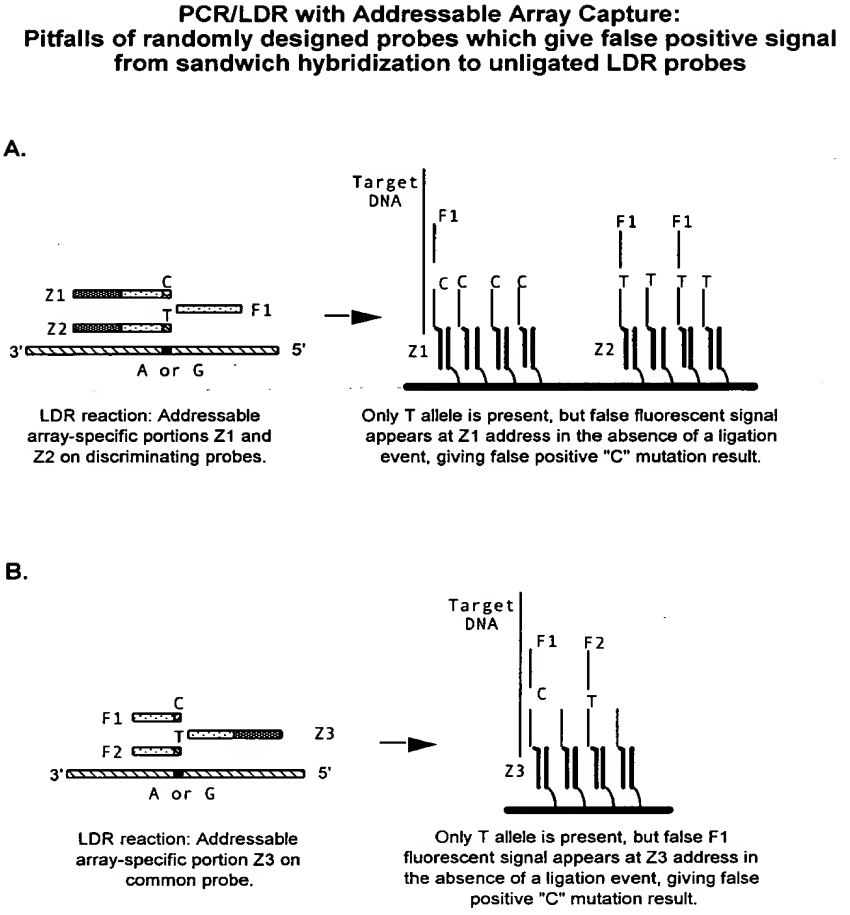


In Figure 2A, the common probe can hybridize somewhat to the Z1 address on the array, giving a false positive signal for the "C' allele. In Figure 2B, the allele specific probe containing the F1 fluorescent group can hybridize somewhat to the Z3 address on the array, giving a false positive signal for the "C' allele. In a typical LDR reaction, 10% to 20% of the LDR probes are converted to product. However, PCR/LDR is very sensitive and can distinguish a single point mutation in a thousand-fold excess of wild-type DNA. If the technique is being used to detect a low abundance mutation, the percent of product generated ranges from 0.01% to 1% of the starting LDR probes. That rare product then competes with unreacted addressable array specific portions for hybridization to the correct address. Even low levels of false hybridization of the fluorescently labeled LDR probe to that or another address may give a false positive result. In a multiplex system, none of the fluorescently

labeled LDR probes should falsely hybridize with any of the addresses on the array. Thus, considerable care is required to properly design the capture oligonucleotide probes to avoid false positive signals.

7. Another problem with random design of the capture oligonucleotide is the potential for sandwich hybridization, because the capture oligonucleotide probe's Tm was too low. For the probes of the present application, stable addressable array-specific portions must be formed at a temperature which is sufficiently above the ligation reaction temperature (65°C). Unless this is achieved, false positive signals may result from hybridization between adjacent unreacted LDR probes which are hybridized to the target. This is illustrated in Figure 3.

Figure 3



In Figure 3A, a sandwich hybridization of unligated common probe and captured "C" allele addressable array-specific portion containing a probe with target DNA giving a false positive signal for the "C' allele. In Figure 3B, a sandwich hybridization of unligated allele "C" probe and the captured probe containing a common addressable array-specific portion with target DNA gives a false positive signal for the "C' allele. The addressable array-specific portion design of the present invention provides calculated T_m values to avoid this problem.

8. Thus, it is important to design the addressable array-specific portion of each LDR probe and the complementary capture oligonucleotides such that the addressable array-specific portions of each oligonucleotide probe hybridizes to its complementary sequences on the addressable array under hybridization conditions where either direct or

sandwich (indirect) hybridization of any labeled LDR probe or LDR product to a non-cognate address is minimized or below the limit of detection.

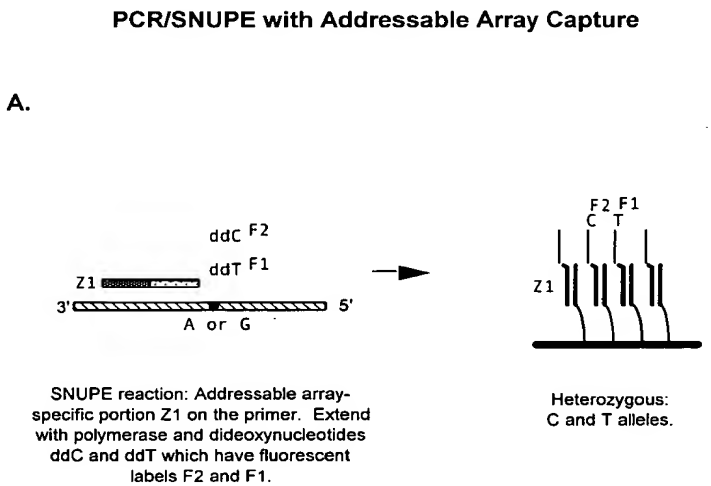
U.S. Patent No. 5,981,176 and WO 93/25563 to Wallace

9. I am familiar with U.S. Patent No. 5,981,176 and WO 93/25563 both to Wallace (collectively "Wallace") and submit this declaration to demonstrate that the method disclosed by Wallace teaches away from the present invention.

10. Wallace relates to a method of detecting and discriminating between nucleic acid sequences. The method utilizes a primer having a 3' portion which is specific for the desired nucleic acid sequence and a 5' portion which is complementary to a preselected nucleic acid sequence. Extension of the 3' portion of the primer with a labeleled deoxynucleoside triphosphate yields a labeled extension product if, but only if, the template includes the target sequence. The labeled extension product is detected by hybridization of the 5' portion to the preselected sequence with the preselected sequence preferably being bound to a solid support along with many other sequences.

11. Figure 4 summarizes the single nucleotide primer extension technique ("SNUPE") using a randomized capture portion, essentially as described by Wallace; however, Wallace only utilizes this technique with radioactively labeled dideoxynucleotides.

Figure 4



In this scheme, a primer specific to the gene and containing a random 20 mer sequence on the 5' end of a target specific portion, hybridizes to the target directly adjacent to the base in question. A non-proofreading polymerase extends the primer with a labeled dideoxynucleotide (in this case, fluorescently labeled F1 or F2). Capture of the extended primer by hybridization at the appropriate address on an array (Z1) and determination of the fluorescent label at that address allows the different alleles to be distinguished.

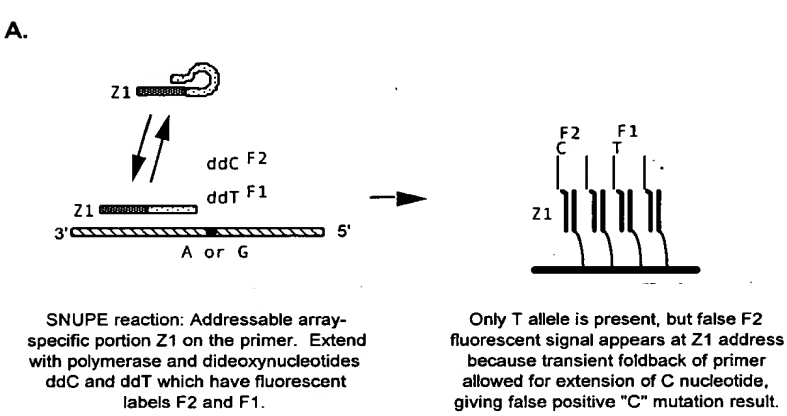
12. A number of the aspects of SNUPE make its features inapplicable to detecting single based differences by the ligase detection reaction ("LDR")/universal array capture technique of the present invention. In general, SNUPE is not adaptable to LDR/universal array capture, because the former uses random primers and achieves target discrimination as a result of polymerase extension while the latter uses an additional probe to distinguish target sequences. The use of polymerase extension SNUPE creates a number of opportunities to general false positive results which would not happen with LDR/universal array capture. In view of this deficiency in SNUPE, one distinguishing nucleic acids with LDR/universal array capture based on single base difference would regard the SNUPE technique as relevant.

13. Firstly, Wallace's process is limited to using the random capture probe on the 5' side. Thus, all the allele signals must come to the same address on the array. Since different fluorophores may overlap with one another, this leads to significant problems when attempting to distinguish multiple potential mutations in a single codon, for example the K-ras G12 codon. While it may be possible to leave out the dideoxynucleotide which would be inserted opposite wild-type DNA, this would work for only one position at a time. Thus, the technique of Wallace is not amenable to multiplex detection of multiple mutations at multiple sites. One cannot place the capture oligonucleotide on the 3' side since polymerases only synthesize in the 5'-3' direction.

14. Secondly, in the process of Wallace, the gene specific primer may form a transient hairpin and be extended by one base which is complementary to its own sequence of the random probe.

Figure 5

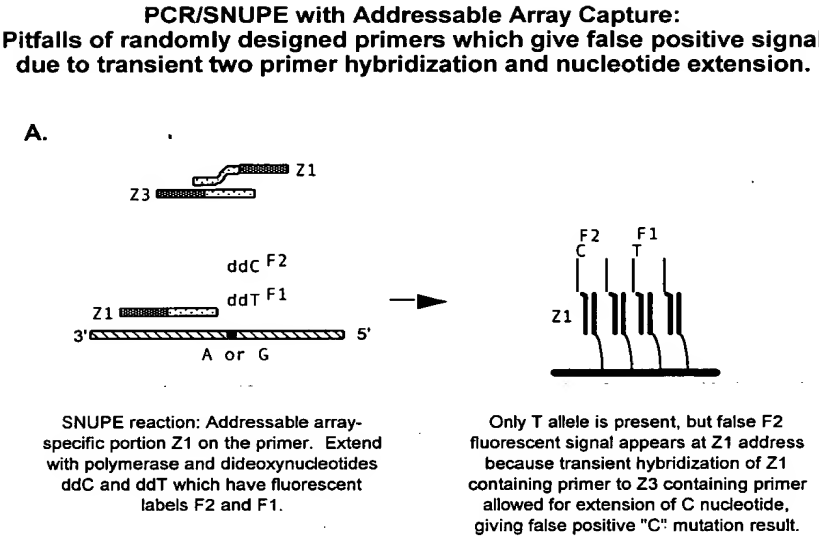
**PCR/SNUPE with Addressable Array Capture:
Pitfalls of randomly designed primers which give false positive signal due to transient primer foldback and nucleotide extension.**



This is shown in Figure 5 where the addressable array-specific portion Z1 of the primer, instead of hybridizing to the target nucleic acid, folds-back, hybridizes to itself, and is extended by polymerase. As a result, if only the nucleic acid with the T allele is present in the sample, the target-specific extension of the primer yields an extension product with the F1 label, while the primer-specific extension may happen to yield an extension product with the F2 label. When captured on the solid support, both of these products are displayed on the support even though no target having the C allele is present in the sample.

15. Thirdly, the process of Wallace is also susceptible to transient hybridization of 2 primers followed by polymerization.

Figure 6



As shown in Figure 6, the primer with an addressable array-specific portion Z1 is intended to be useful in detecting T or C alleles in the target nucleic acid. However, in cases of multiplex detection, where a different primer having an addressable array-specific portion Z3, is also present to detect a different allele in another target sequence, those primers may hybridize to another and be extended by a labeled dideoxynucleotide other than what they would otherwise be extended with if hybridized to the target nucleic acid sequence. As shown in Figure 6, if only the nucleic acid with the T allele is present in the sample, the target-specific extension of the primer yields an extension product with the F1 label, while the primer-specific extension may happen to yield an extension product with the F2 label. When captured on the solid support, both of these products are displayed on the support even though no target having the C allele is present in the sample.

16. The susceptibility of Wallace to false positive detection due to its use of randomly designed capture probes (where the sequence numbers are set forth in Wallace) is further demonstrated by the teachings of Wallace as follows:

A. Random capture sequences which form hairpins to themselves.

TCAGTTACCACGGTAACTGG This random sequence has 8 base inverted repeat, forms hairpin, and will not hybridize well to capture oligonucleotide probe on an addressable array.

B. Random capture oligonucleotide probe sequences which hybridize to each other.

CATAATGCGGTCTCGATACG Seq ID# 8

CAAATGCGGTCTCGATATCG This slight modification of sequence # 8 has an 8 base repeat on the end and will hybridize to another molecule during the coupling reaction and, therefore,will not hybridize to an addressable array-specific portion.

CAAATGCGGTCTCGATATCG
GCTATAGCTCTGGCGTAAAC

C. Random capture sequences which are similar, allowing for hybridization and capture at the wrong address.

ATGGGCTCCTGCGTAAATCA Seq ID # 2
AGTGCGCTCTCTTGAGCAAA Seq ID # 3

ATGCGCTCTCCTGGGTAAAA Modified Seq ID # 17,
AGTGCGCTCTCTTGAGCAAA Seq ID # 18. Slight modification shows high degree of similarity between oligonucleotides (16/20) allowing for false hybridization at incorrect addresses.

D. Random capture sequences which hybridize with any other LDR probe in the LDR mixture, thus precluding correct hybridization on the array (false-negative) or allowing for sandwich or indirect hybridization to the array resulting in false-positive signal.

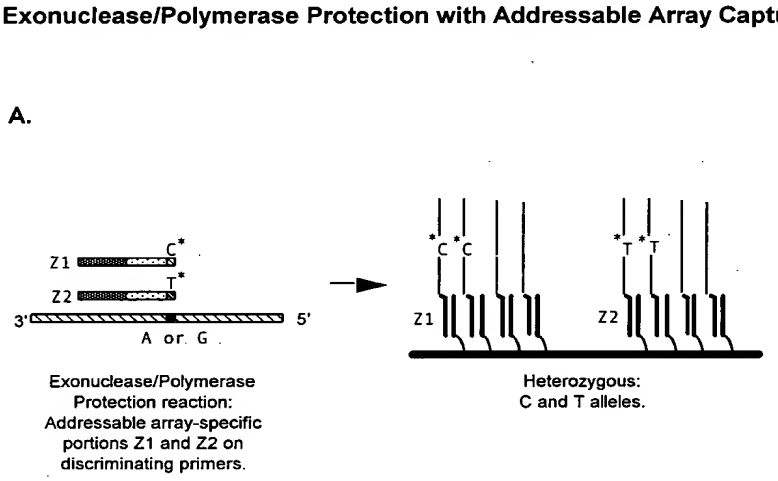
17. As shown above, the SNUPE process is susceptible to detection of false positive results due to its efforts to discriminate target nucleic acid sequences by use of random primers and polymerase extension. This problem is worsened when the SNUPE process is preceded by PCR, because, in that case, all dNTPs from that earlier reaction must be destroyed to prevent SNUPE primer extension before an incorrect dideoxynucleotide is inserted at a downstream base. In addition, SNUPE cannot detect small insertions, deletions, or slippage events in mono- or di-nucleotide repeats. By contrast, the LDR/universal array

capture technique is able to detect such conditions with minimal risk of false signals by careful design of both the oligonucleotide probes used for LDR and the oligonucleotide probes used for array capture. As a result, the features of the SNUPE process would have little applicability to the LDR/universal array capture technique.

U.S. Patent No. 5,391,480 and WO 90/11272 both to Davis, et al.

18. Davis discloses a method of detecting a nucleotide at a specific location within a nucleic acid using exonuclease activity.

Figure 7



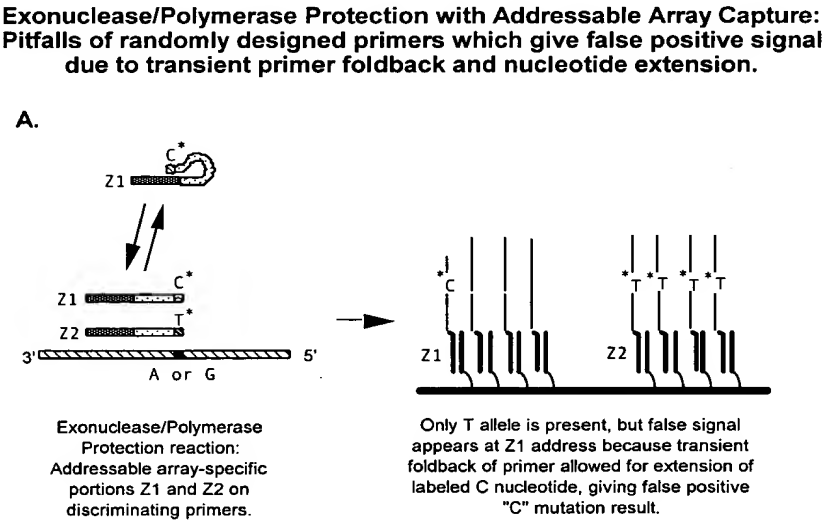
In particular, as shown in Figure 7 of Davis, a test sample of DNA is treated with a labelled oligonucleotide having a primer portion capable of hybridizing to target DNA, if present in the test sample. The label is attached to a nucleotide in the primer portion at a test position and that nucleotide will hybridize to the target DNA if there is complementarity at that position. On the other hand, if such complementarity is lacking, the labelled nucleotide will not be bound. As a result, a subsequent exonucleolytic treatment step, which follows a polymerase extension procedure, will excise the label if complementarity is absent at the test position. The primer includes a tail portion (Z1 and Z2 in Figure 7) so that the primer can be immobilized on a solid support and detected when the primer is contacted with the solid support under conditions effective to hybridize the tail portion to a complementary

oligonucleotide on the solid support. In Figure 7, since the sample is heterozygous for target nucleic acids with the C and T alleles, labeled primers are captured at both the Z1 and Z2 addresses.

19. A number of aspects of the Davis process make its features inapplicable to detecting single base differences by LDR/universal array capture. The Davis process is not adaptable to LDR/universal array capture, because the former is susceptible to false positive detection as a result of its use of hybridization, exonucleolytic treatment, and polymerase extension. Since these opportunities for the Davis process to detect false positives are not present in LDR/universal array capture, the Davis process would not be viewed as relevant to LDR/universal array capture.

20. Firstly, in the process of Davis, the gene specific primer may form a transient hairpin and be extended by several bases which are complementary to its own sequence and/or the random probe sequence.

Figure 8



This is shown in Figure 8 where the addressable array-specific portion Z1 of the primer, instead of hybridizing to the target nucleic acid, folds-back, hybridizes to itself, and is extended by polymerase. As a result, if only the nucleic acid with the T allele is present in the sample, the target-specific extension of the primer yields an extension product with a

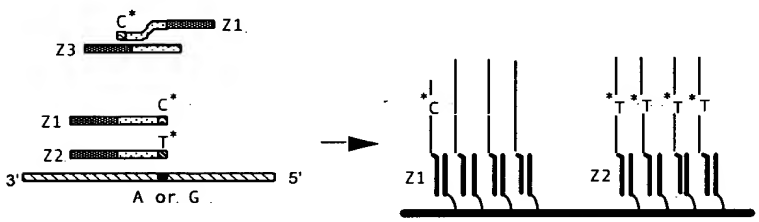
label at the Z2 address, while the primer-specific extension may happen to yield an extension product with a label at the Z1 address. When captured on the solid support, both of these products are displayed on the support even though no target having the C allele is present in the sample.

21. Secondly, the process of Davis is also susceptible to transient hybridization of 2 primers followed by polymerization.

Figure 9

Exonuclease/Polymerase Protection with Addressable Array Capture: Pitfalls of randomly designed primers which give false positive signal due to transient two primer hybridization and nucleotide extension.

A.



Exonuclease/Polymerase Protection reaction: Addressable array-specific portions Z1 and Z2 on discriminating primers.

Only T allele is present, but false signal appears at Z1 address because transient hybridization of Z1 containing primer to Z3 containing primer allowed for extension of labeled C nucleotide, giving false positive "C" mutation result.

As shown in Figure 9, the primer with an addressable array-specific portion Z2 is intended to be useful in detecting the T allele in the target nucleic acid. However, in cases of multiplex detection, where a different primer having an addressable array-specific portion Z2, is also present to detect nucleic acids with the C allele, those primers may hybridize to another and be extended by polymerase so that a label which should have been removed by exonucleolytic treatment remains in place. As shown in Figure 9, if only the nucleic acid with the T allele is present in the sample, the target-specific extension of the primer with the Z2 portion yields a labelled extension product, while the primer-specific extension may happen to yield an extension product with a labelled extension product which is produced when target nucleic acid with the C allele (which is absent here from the sample being

analyzed) is present. When captured on the solid support, both of these products are displayed on the support even though no target having the C allele is present in the sample.

22. As shown above, the Davis process is susceptible to detection of false positive results due to it efforts to discriminate target nucleic acid sequences by use of primers suitable for hybridization, exonucleolytic primers, and polymerase extension. The Davis process starts with labeled probe which is subsequently destroyed by exonuclease digestion. Thus, if exonuclease digestion is incomplete, then the low level of false positive signal would be mistakenly interpreted as indicating the presence of low level mutant target. In addition, the Davis process cannot detect small insertions, deletions, or slippage events in mono- or di-nucleotide repeats. By contrast, the LDR/universal array capture technique is able to detect such conditions, including low levels of mutant target, with minimal risk of false signals by careful design of both the oligonucleotide probes used for LDR and the oligonucleotide probes used for array capture. As a result, the features of the Davis process would have little applicability to the LDR/universal array capture technique.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 1/22/01

Dr. Francis Barany

Francis Barany